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Structural and mechanistic insights into prokaryotic energy-coupling factor transporters

Dirk J. Slotboom

Abstract | Energy-coupling factor (ECF) transporters belong to the ATP-binding cassette (ABC)-transporter family and mediate the uptake of essential micronutrients in many prokaryotic species. Two crystal structures of bacterial ECF transporters have recently been obtained and suggest that transport involves an unprecedented re-orientation of a membrane protein in the lipid bilayer during catalysis. In this Progress article, I present the new structural insights, discuss a testable model for the transport mechanism and consider the more general implications of these findings for our understanding of membrane transporters.

The term energy-coupling factor (ECF) transporter was coined in the 1970s to describe transporters that are used by lactic acid bacteria for the uptake of vitamins[1–5]. Initial studies showed that these transporters depend on two components: a membrane-bound protein that is specific for each substrate (now known as an S component or EcfS) and a common energy-coupling factor. This early work also showed that the energy for transport was supplied by the hydrolysis of ATP[2]. These observations date from the pre-genomics era and, although some of the binding proteins were purified and their amino acid compositions determined[3,4], the molecular identity of the transport systems remained elusive until the 2000s[6–14].

It is now evident that ECF transporters are present in approximately 50% of prokaryotic species, and that they catalyse the cellular uptake of a range of micronutrients, including water-soluble vitamins (such as riboflavin and thiamin) and their precursors, as well as transition metal ions (such as Ni^{2+} and Co^{2+})[6] (TABLE 1). ECF transporters are particularly abundant in the Firmicutes phylum of Gram-positive bacteria (TABLE 1), many members of which are human pathogens. ECF transporters are often indispensable proteins in these pathogens as they lack the complete biosynthesis pathways for the transported compounds. For example, *Listeria monocytogenes* lacks the ability to synthesize thiamin and depends on an ECF transporter for the uptake of this essential micronutrient[15]. In other clinically relevant bacteria, such as *Mycoplasma genitalium, Streptococcus pneumoniae* and *Staphylococcus aureus*, the genes that encode ECF transporters are also essential[16–20], probably because these organisms lack the pathways for folate, biotin and thiamin biosynthesis, respectively (Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database). These examples highlight the importance of ECF transporters and suggest that the proteins involved are potentially useful targets for novel antibiotics.

Similarly to other membrane transport proteins, delineating the mechanistic details of transport requires high-resolution crystal structures and the structures of two *Lactobacillus brevis* ECF transporters have recently been obtained[21,22]. In this Progress article, I describe the genetic organization, overall stoichiometry and structural composition of ECF transporters and discuss a novel mechanism of transport on the basis of the recent structural insights.

ECF transporters: the basics

ECF transporters consist of two parts: a substrate-binding S component and an ECF module (FIG. 1). S components are small integral membrane proteins (M_w ~20 kDa) that provide substrate specificity to the ECF transporter. So far, 21 different S component families have been identified, each of which is specific for a distinct substrate[3,4,6] (TABLE 1). Although S components for different substrates lack a high degree of sequence similarity (they have on average 10–20% sequence identity), crystal structures of isolated S components have shown that their global structures are conserved[23–25]. It is likely that this fold is shared by all S components (with the possible exception of the S components for Co^{2+} and Ni^{2+}; BOX 1). A conserved functional feature of S components is their high affinity for substrates, with dissociation constants in the low to sub-nanomolar range[9,11,24,26].

The vast majority of S components associate with an ECF module that consists of three proteins: an integral membrane protein, EcfT (also known as the T component), and two similar or identical cytosolic ATPases, EcfA and EcfA′ (also known as A components) (FIG. 1). Although the amino acid sequences of EcfT proteins are highly diverse, they can be identified by two short conserved Ala-Arg-Gly motifs (see below)[27]. The two ATPases of the ECF module belong to the large family of ATPases (also known as nucleotide-binding domains (NBDs)) that are found in ATP-binding cassette (ABC) transporters[28]; thus, ECF transporters form a branch of this large superfamily of transporters (BOX 1). Similarly to other ABC transporters, ECF transporters use the free energy that is released by ATP hydrolysis to transport substrates into cells[2,29].

Diversity among ECF transporters. ECF transporters are classified into two distinct groups (known as group I and group II) on the basis of the chromosomal location of the genes that encode the S components and ECF modules[6] (FIG. 1a, b). Some organisms only contain ECF transporters that belong to one group, but others, in particular the Firmicutes, contain transporters from both groups[6].

In group I, the genes that encode the ECF module and the S component are located in the same operon, and the four proteins

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Table 1 | **Substrate specificity and distribution of ECF transporters**

S component	Substrate*‡	Group*	Prokaryotic distribution*
BioY	Biotin (vitamin B_7)	I, II and solitary	Actinobacteria; Archaea; *Chlamydia* spp.; Cyanobacteria; *Deinococcus* spp.; Firmicutes; Fusobacteria; Proteobacteria; Spirochaetes; Thermotogae; *Thermus* spp.
CbiMN	Co^{2+}	I	Actinobacteria; Archaea§; Bacteroidetes; Cyanobacteria; Firmicutes§; Proteobacteria; Spirochaetes; Thermotogae
CblT	Dimethylbenzimidazole‡	Mostly II	Firmicutes
CbrT	Cobalamin (vitamin B_{12})‡	I and II	Actinobacteria§; Archaea; Firmicutes§
FolT	Folates (vitamin B_9)	II	Firmicutes§; Themotogae
HmpT or PdxU2¶	Hydroxymethylpyrimidine or pyridoxine‡	Mostly II	Archaea; Firmicutes§; Thermotogae§
HstT	Unknown	Mostly I	Actinobacteria; Archaea; Firmicutes; Spirochaetes
LipT	Lipoate‡	II	Firmicutes
MtaT	Methionine precursor‡	I	Actinobacteria; Archaea; Firmicutes
MtsT	S-adenosylmethionine‡	I	Archaea; Firmicutes§; Proteobacteria
NiaX	Niacin (vitamin B_3)	II	Firmicutes
NikMN	Ni^{2+}	I	Actinobacteria; Archaea§; Bacteroidetes; Cyanobacteria; Firmicutes; Proteobacteria§; Spirochaetes
PanT	Pantothenate (vitamin B_5)	Mostly II	Actinobacteria; Firmicutes§; Themotogae
PdxU	Pyridoxine (vitamin B_6)‡	Mostly II	Actinobacteria; Archaea§; Firmicutes§;Thermotogae§
QueT	Queuosine or precursor‡	Mostly II	Actinobacteria; Firmicutes§; Thermotogae
QrtT	Queuosine or precursor‡	I and II	Firmicutes; Thermotogae; Actinobacteria; Proteobacteria
RibU	Riboflavin (vitamin B_2)	Mostly II	Actinobacteria; Archaea; Firmicutes§; Thermotogae§
ThiT	Thiamin (vitamin B_1)	II	Firmicutes
ThiW	Thiazole‡	I and II	Actinobacteria; Archaea; Chloroflexi; Firmicutes
TrpP	Tryptophan	I and II	Archaea; Firmicutes
YkoE	Hydroxymethylpyrimidine‡	I	Actinobacteria§; Archaea; Firmicutes§

*Data taken from REFS 6,27,29. ‡ These substrates are only predicted to be transported by the corresponding S component. §The phyla in which the indicated S components are most abundant. ¶This family of S components was originally annotated as HmpT[6] but was later renamed PdxU2 because genome context analysis showed that the proteins might be specific for pyridoxine[27].

are predicted to form a dedicated complex. In some cases, two or three components of the complex are fused into a multi-domain protein, resulting in complexes that consist of three or two subunits, rather than four (FIG. 1a). Some organisms encode several group I ECF transporters, and this feature is most pronounced in archaea (for example, *Thermofilum pendens* contains six transporters) and Actinobacteria[6,27]. The cellular levels of group I transporters are often regulated according to substrate requirements, such that the transporters are highly expressed when the cytoplasmic concentrations of corresponding substrates are low and their expression is downregulated when the substrate is abundant[6]. Regulation of ECF transporter expression occurs at the transcriptional and translational levels. In many cases, riboswitches are used to regulate the expression of ECF transporters. Riboswitches are sequence motifs that bind metabolites and are found upstream of the coding region in several mRNAs[30]. Binding results in alteration of the mRNA structure, which affects transcription or translation. In the case of ECF transporters, the encoding mRNA adopts a conformation that is incompatible with translation when the transported substrate is bound (that is, when substrate levels are high), whereas the substrate-free riboswitch enables translation to proceed[6].

The S component genes of group II transporters are not located in the same operon as the genes for the ECF module, and multiple different S component genes are usually scattered around the chromosome. These S components, which differ in their substrate specificity, share the same ECF module[6,29]. Thus, the same ECF module can associate with distinct S components and thereby power the import of chemically diverse substrates; for example, the ECF module of the group II ECF transporter in *L. brevis* associates with seven different S components that transport seven different substrates (FIG. 1b). As different S components lack a high degree of sequence similarity, the use of the same ECF module by group II transporters raises an intriguing question: how does the same ECF module recognize unrelated S components? The recent crystal structures[21,22] provide some clues to explain how this might be possible (see below).

The ECF modules of group II transporters are usually constitutively expressed[6]. By contrast, expression of the different S components is regulated, often by riboswitches in the encoding mRNAs[6,10,13] (see above). This enables the cellular pool of S components to increase substantially when the corresponding substrate is lacking[5,6,10]. As a result, the number of ECF modules in a cell may become limiting, resulting in a surplus of S components that do not have an ECF-module partner. A potential role for these lone S components could be to scavenge scarce micronutrients from the

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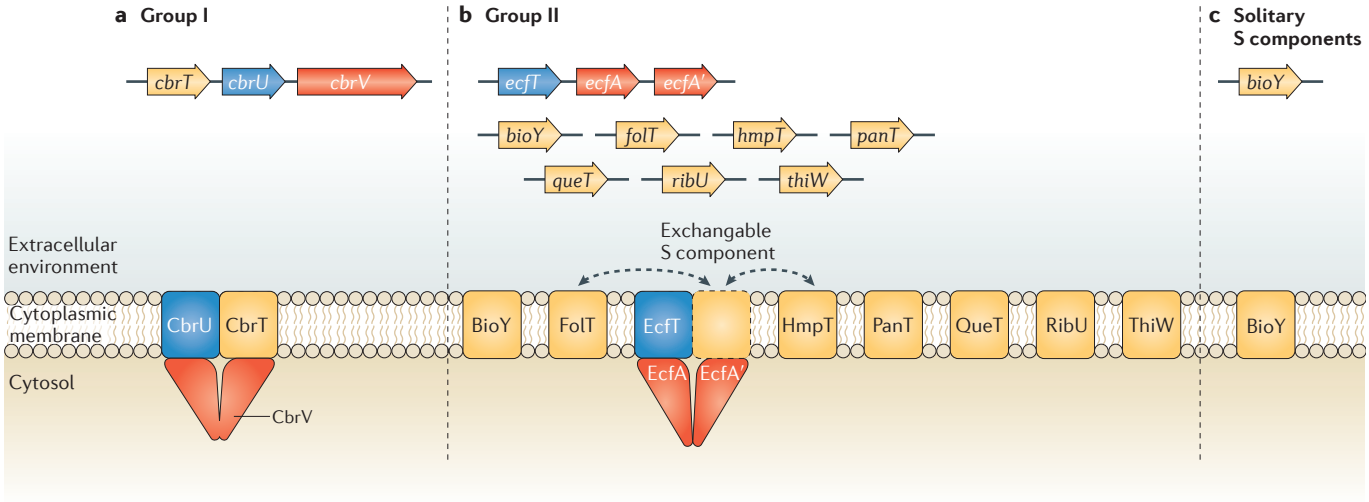

Figure 1 | **Composition and architecture of ECF transporters. a** | The genetic organization and subunit composition of the *Lactobacillus brevis* group I energy-coupling factor (ECF) transporter complex CbrTUV. CbrT is the substrate-binding S component, and CbrU (which is the EcfT component) and CbrV (which consists of two fused EcfA subunits) constitute the ECF module. In contrast to group II transporters, the genes that encode these components are found in the same operon, and they form a complex that is dedicated to the transport of one specific substrate (in this case, cobalamin). **b** | The *L. brevis* group II ECF transporter contains seven different S components (BioY, FolT, HmpT, PanT, QueT, RibU and ThiW), and the genes that encode these are scattered around the chromosome. All of the S components interact with a shared ECF module (EcfAA′T). **c** | Some prokaryotes encode the biotin-specific S component BioY, which can function independently of an ECF module.

environment and keep them tightly bound until an opportunity arises to associate with an ECF module, which would then enable the transport of the scavenged substrate into the cell.

In addition to group I and group II transporters, there are also solitary S components that seem to function independently of an ECF module, although these transporters are rare. For example, a few bacteria (including all sequenced Chlamydia species, with the exception of *C. pneumoniae*, and many diverse proteobacterial and cyanobacterial species) contain the S component for biotin, BioY, but lack an ECF module[6,31,32] (FIG. 1c). The mechanism of transport that is used by the solitary BioY proteins is unknown, but the structures of the complete ECF complexes[21,22] might provide some clues (see below). It has also been reported that the S components of two different *Rhodobacter capsulatus* group I transporters (BioY and the bipartite S component for cobalt CbiMN) are able to mediate transport in the absence of their respective ECF modules (BioMN and CbiQO, respectively)[8]. Whether such transport is physiologically relevant is unclear as all group I transporters have a dedicated ECF module. In addition, although the *R. capsulatus* BioY transporter was shown to be active in recombinant *Escherichia coli* cells, it has not been possible to confirm this transport activity using the purified protein reconstituted in liposomes[24].

Structural overview

Both of the *L. brevis* ECF transporters that were recently crystallized belong to group II and consist of the same ECF module but contain different S components[21,22]. One complex (ECF–FolT) contains FolT, which is the S component that transports folates[21]; and the other complex (ECF–HmpT) contains thiamine precursor transporter HmpT[22], which is the S component that was originally predicted to transport hydroxymethyl pyrimidine[6]. HmpT has been renamed PdxU2 as genomic context analysis suggests that it transports pyridoxin (The SEED database[27]). Here, the name HmpT is used to be consistent with the use of this term in the crystallography study[22].

The structures were solved to medium resolution (3.0 Å for ECF–FolT and 3.6 Å for ECF–HmpT), with reasonable electron density for the α-helical segments but poorer (or missing) density for the loop regions. Therefore, caution needs to be taken when deducing a mechanism of transport from the structures as they do not provide insight at the level of atomic details. Both ECF–FolT and ECF–HmpT were crystallized in a similar conformational state, with neither nucleotides (ADP or ATP) nor transported substrates (folates, hydroxymethylpyrimidine or pyridoxin) bound. The structures provide tantalizing clues on how ECF transporters might function, although it should be emphasized that it is not possible to deduce a complete transport mechanism from a single conformational state.

The stoichiometry of the complexes. The two complexes share a similar overall structure, and the four subunits (EcfA, EcfA′, EcfT and the S component (FolT or HmpT)) are present in a 1/1/1/1 stoichiometry[21,22] (FIG. 2a). This stoichiometry had previously been shown using biophysical analysis of other group II transporters[29]. A 1/1/1/1 stoichiometry had also been inferred from information about gene fusions in several organisms[6]. The two ATPases are sometimes fused into a two-domain protein (FIG. 1a). Similarly, the S component can be fused to EcfT, and either the S component or EcfT can be fused to the two ATPases, forming three-domain proteins. If subunit stoichiometry is conserved among all ECF transporters, these fusions are only compatible with a 1/1/1/1 stoichiometry. Therefore, it is likely that the 1/1/1/1 subunit stoichiometry is the basic structural unit of ECF transporters.

However, it should be noted that a different subunit stoichiometry (1/1/2/2 for EcfA/EcfA′/EcfT/S component) has been proposed on the basis of crosslinking studies of purified group II ECF complexes[33]. This discrepancy might be explained by the tendency of purified membrane proteins to form non-specific aggregates, so it is possible that these crosslinked complexes were an artefact. The 1/1/1/1 stoichiometry also differs from the stoichiometry that

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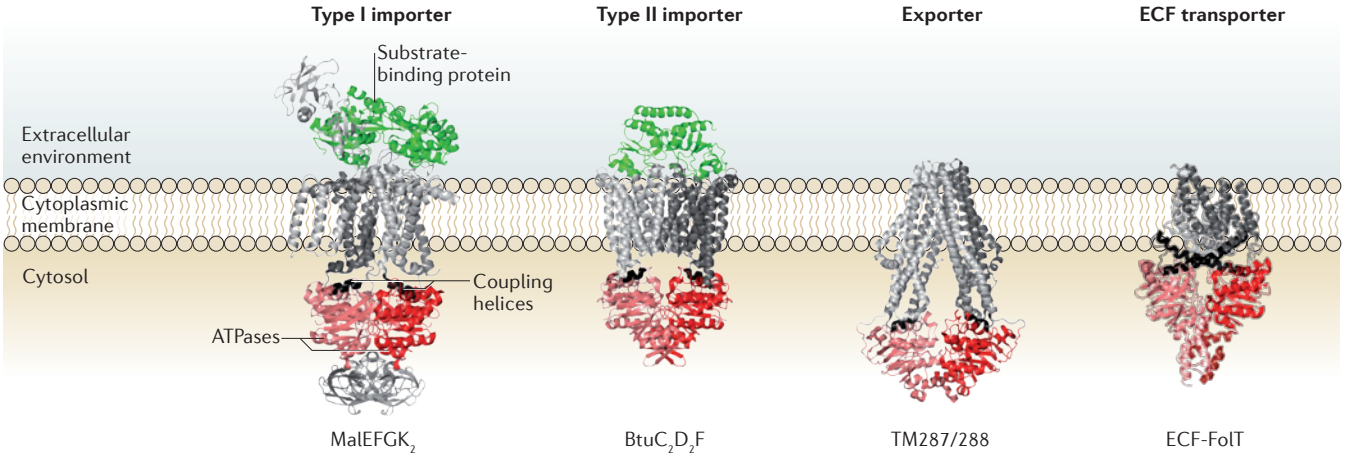
Box 1 | **Diversity of ABC transporters**

Energy-coupling factor (ECF) transporters belong to the ubiquitous class of ATP-binding cassette (ABC) transporters (see REF. 28 for a recent review), all members of which contain two conserved cytosolic nucleotide-binding domains (NBDs) or subunits that bind and hydrolyse ATP, and are associated with a pair of integral membrane subunits. In contrast to the ATPases, the structures of the integral membrane subunits are not universally conserved among ABC transporters. Four different structural classes have been discovered, which probably have different mechanisms of substrate translocation (see the figure): type I importers (which are exemplified by the maltose transporter $MalEFGK_2$ from *Escherichia coli*[57–61]; Protein Data Bank (PDB) accession 2R6G); type II importers (exemplified by vitamin B_{12} transporter $BtuC_2D_2F$ from *E. coli*[62–64]; PDB accession 4FI3); exporters (such as multidrug and peptide transporters[65–67], exemplified by the drug exporter TM287/288) from *Thermotoga maritima*; PDB accession 3QF4); and ECF transporters (such as ECF–FolT from *Lactobacillus brevis* for folate transport[21]; PDB accession 4HUQ). Type I and II importers and energy-coupling factor (ECF) transporters are only found in prokaryotes, whereas exporters are found in all kingdoms of life[28].

In addition to the two ATPases and membrane subunits, several other proteins can associate with ATP transporters. Notably, type I and II importers are dependent on water-soluble substrate-binding proteins or domains that provide substrate specificity (reviewed in REF. 68). Exporters do not require additional proteins for substrate recognition. ECF importers do not make use of soluble binding proteins, but use one of the integral membrane subunits (the S component) instead. In most cases, S components contain all of the determinants that are required for substrate binding, with the exception of the S components that bind Ni^{2+} and Co^{2+} (NikM and CbiM, respectively). Both of these metal-binding S components seem to require auxiliary small integral membrane proteins for substrate binding — CbiN for CbiM, and either NikN or NikKL (which is a complex of two proteins) for NikM[6,69,70] — but the exact role of these additional proteins remains to be elucidated. CbiM and NikM have seven predicted membrane-spanning α-helices, in contrast to the common six helices of other S components[23–25]. Whether CbiM and NikM share the conserved structural core of the other S components is not clear.

In ABC transporters, the binding and hydrolysis of ATP leads to conformational changes in the ATPase subunits, which are then transmitted to the membrane subunits. The coupling helices, which are α-helical structures on the cytoplasmic side of the membrane subunits, contact the ATPase subunits and mediate the transmission of the conformational changes to the transmembrane components (see the figure). In contrast to ECF transporters, exporters and type I and II importers translocate the substrate through the interface between the transmembrane subunits. The conformational changes in the transmembrane subunits trigger cavity access to alternate between either the intracellular or the extracellular milieu. In ECF transporters, the substrate binds to the S component, and the conformational changes that occur as a result of ATP binding and hydrolysis probably lead to the toppling of the S component (see main text).

The two membrane subunits in type I and type II importers and in exporters are either identical or have similar folds, making these ABC transporters (pseudo-)symmetrical. By contrast, the two membrane proteins in ECF transporters (the S component and EcfT) are unrelated; thus, these proteins have an asymmetrical conformation.



was proposed from *in vivo* experiments on the group I biotin transport ATP-binding BioMNY transporter from *R. capsulatus*[34–36], which indicate the presence of two or more EcfT subunits and S components per complex. Although it is possible that these stoichiometric discrepancies are the result of genuine structural divergence between different ECF transporters, another explanation is that two or more complexes that have 1/1/1/1 stoichiometry cluster together in the membrane, giving rise to apparently different stoichiometries. If clustering does occur, it is still probable that the 1/1/1/1 complex is the basic structural unit of all ECF transporters.

The S component topples over. The most remarkable feature of the structures[21,22] is the orientation of the S components in the complexes. Integral membrane proteins generally have a fixed orientation in the lipid bilayer, in which hydrophobic segments (usually α-helices) zig-zag through the membrane and hydrophilic loops protrude into the aqueous environment on either side of the membrane (for a review of membrane protein topology, see REF. 37). Whether a loop is located on the cytosolic or the extracellular (periplasmic) side of the membrane is determined by the positive-inside rule: intracellular loops tend to be richer in the positively charged residues arginine and lysine than extracellular loops[38].

Molecular dynamics simulations of the solitary S component for thiamin ThiT, of which a crystal structure had been determined before the structures of the complete ECF transporters, are consistent with these generic features of membrane proteins[25,39] (FIG. 2b). The protein contains six hydrophobic α-helices that traverse the hydrophobic core of the bilayer, and the connecting loops are alternately located on the cytosolic and extracellular (or periplasmic) side of the membrane. The substrate, thiamin, binds to a site that is located close to the extracellular side of the membrane. The solitary S components BioY and RibU (S component for riboflavin) are predicted

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to have the same orientation in the membrane as ThiT[23,24].

Unexpectedly, the orientation of the S components FolT and HmpT in the complete ECF transporters is entirely different, even though their global folds are the same as RibU, ThiT and BioY[21–25] (FIG. 2c). In the ECF complexes, the S components have 'toppled over' by almost 90 degrees and transmembrane α-helices 1–4 are approximately parallel to the membrane plane, instead of in the perpendicular orientation that is adopted by the solitary S components (FIG. 2b). This parallel orientation is unprecedented for membrane proteins and would have been very difficult to predict as exposure of the hydrophilic loops to the hydrophobic core of the bilayer is energetically unfavourable. However, in the context of the whole ECF complex, the S components still conform to the energetic rules for membrane protein stability. Most loop regions are either located on the cytosolic side of the membrane (obeying the positive-inside rule) or buried at the interface with EcfT, thereby avoiding unfavourable interactions with the hydrophobic core of the membrane. Remarkably, whereas the substrate-binding sites in the solitary S components RibU, ThiT and BioY are located close to the extracellular side of the membrane (FIG. 2b), the toppled S components in the ECF complexes now expose their binding sites — which, in the crystallized complexes, are free of substrates — to the cytosol. This suggests that the re-orientation of the S component could be part of the catalytic cycle, corresponding to the substrate having moved from the extracellular side to the cytosolic side of the membrane, which needs to occur during import.

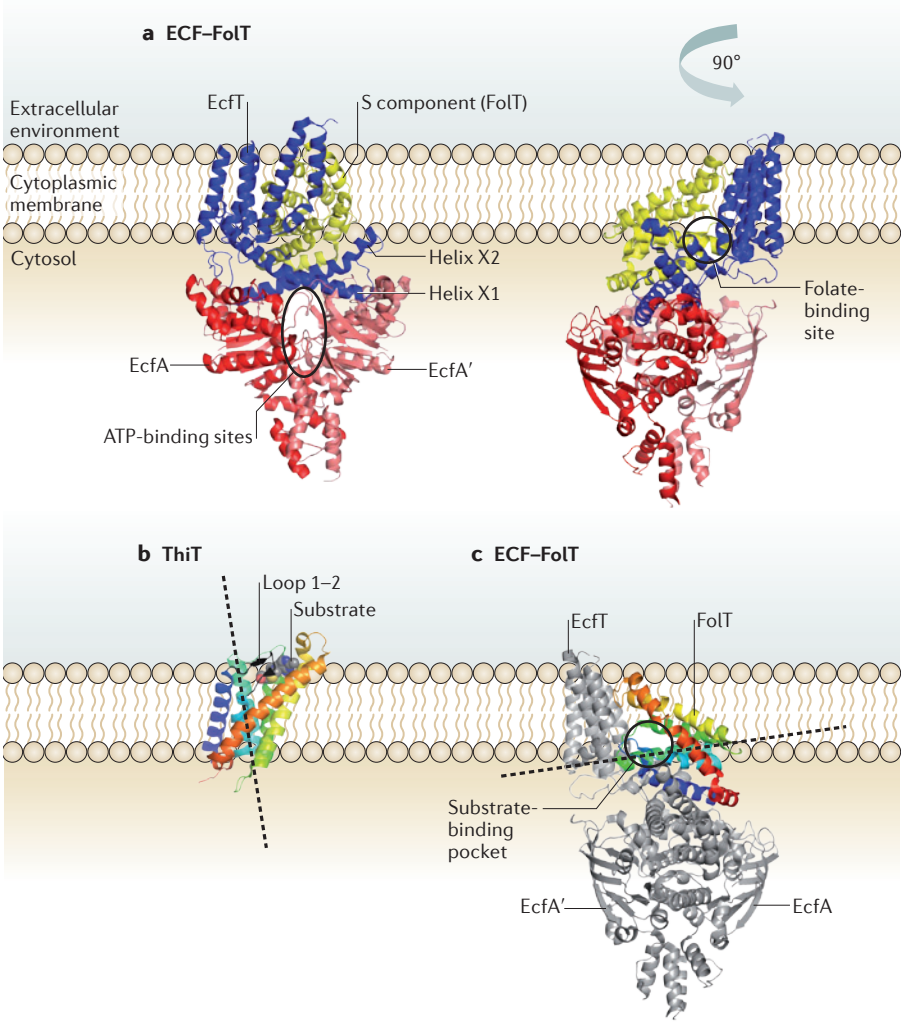

Figure 2 | **Structure of ECF transporters. a** | Structure of energy-coupling factor folate transporter ECF–FolT from *Lactobacillus brevis* in ribbon representation[21] (Protein Data Bank (PDB) accession 4HUQ) from two different viewpoints. The S component FolT provides specificity for folates; the EcfA and EcfA′ subunits bind and hydrolyse ATP and transmit conformational changes to EcfT (via the X1 and X2 helices), which in turn leads to substrate translocation by the S component. **b**,**c** | Ribbon representations of the solitary S component ThiT[25] (PDB accession 3RLB; part **b**) and ECF–FolT[21] (part **c**) in the membrane. The ECF module in part **c** is shown in grey and the S components in parts **b** and **c** are coloured in rainbow from blue (amino terminus) to red (carboxyl terminus). The dashed lines indicate the orientations of α-helix 3 in the solitary ThiT S component (**b**) and in the complete ECF–FolT complex (**c**), highlighting that the S component adopts a distinct membrane orientation in each structure. Compared to ThiT (**b**), in which the α-helices have an orientation that is roughly perpendicular to the membrane plane, FolT has toppled over by approximately 90 degrees and the α-helices are oriented parallel to the membrane plane. The orientation of ThiT in the membrane is consistent with molecular dynamics simulations[39] and shows that the substrate-binding site is located close to the extracellular side of the membrane, whereas in the ECF–FolT complex, this site is accessible from the cytosolic side of the membrane. The loop between transmembrane helices 1 and 2 in ThiT (loop 1–2) functions as a gate over the substrate-binding site.

Transport mechanism

The 'moving-carrier' mechanism. Protein-mediated solute transport across membranes requires that access to the substrate-binding site alternates between the extracellular (or periplasmic) side and cytosolic side of the membrane. This 'alternating access' model was proposed almost half a century ago, long before structures of membrane transporters were solved[40,41], and applies to all membrane transporters, not just to the ABC transporter family. The many crystal structures that are now available for different transport proteins provide insights into the molecular mechanisms that mediate alternating access (recently reviewed in REFS 28,42,43). Three models for transport have been proposed — the rocker-switch mechanism, the gated-pore mechanism and the elevator mechanism (FIG. 3 a–c) — and the ECF transporter structures now suggest that there is a fourth mechanism (FIG. 3d). The basic principles of these models are briefly discussed below, but it is important to note that the mechanism that is used by a single protein can incorporate features from different models.

In the rocker-switch mechanism (FIG. 3a), the substrate binds to the transporter at a site that is located roughly at the centre of the membrane (midway between the extracellular and cytosolic surfaces of the bilayer), which it reaches by diffusing through an aqueous cavity in the protein that is only accessible from one side of the membrane

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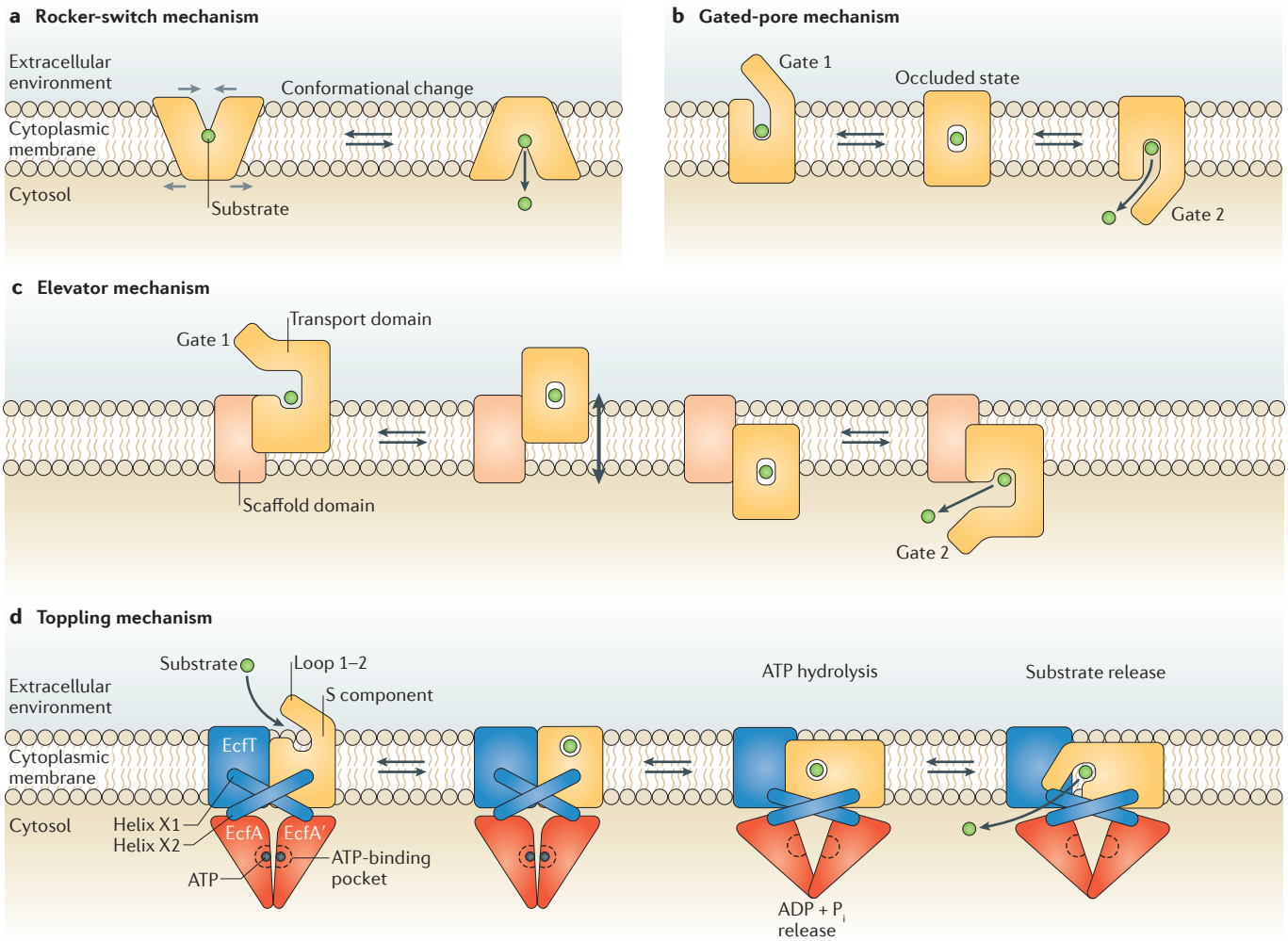

Figure 3 | **Models for alternating-access mechanisms of transport.** **a** | Rocker-switch mechanism. The substrate binds to a site in the protein that is located close to the centre of the membrane. A swivelling movement around a hinge region at the substrate-binding site (light grey arrows) alternates access between the extracellular and cytosolic sides of the membrane. The proton-coupled lactose transporter LacY is a prominent example of a protein that uses this transport mechanism[71]. Exporters of the ATP-binding cassette (ABC) transporter family might also use a similar mechanism[66,67]. **b** | Gated-pore mechanism. The substrate-binding site is located midway in the membrane and alternating access is achieved by the use of two gates. An occluded intermediate state exists when both gates are closed. Ion-coupled transporters such as LeuT (which is a Na^+-coupled amino acid transporter) use this mechanism[43], and type II ABC importers also incorporate elements of the gated-pore mechanism[64]. **c** | Elevator mechanism. Two gates control access to the binding site, and the protein domain that has bound the substrate undergoes a translational movement in the membrane when it is in an occluded state. A scaffold domain is needed to facilitate the elevator-type movement of the transport domain. The Na^+-coupled aspartate transporter Glt_{Ph} is the prototypical example of a transporter that uses this mechanism[72]. **d** | Hypothetical model for ATP-driven transport by ECF transporters using the toppling mechanism. Binding of ATP is proposed to bring the two ATPases into close proximity, and pinches the X-shaped helices (known as X1 and X2) of EcfT together. This is proposed to orient the S component so that its substrate-binding site faces the extracellular environment. The hydrolysis of ATP and release of inorganic phosphate (P_i) and ADP lead to toppling of the S component and the substrate-binding site becomes oriented towards the cytosol, as was observed in the crystal structures of ECF–FolT and ECF–HmpT[21,22].

(for example, the extracellular side). The protein then undergoes a conformational change, in which it swivels around a hinge that is located at the substrate-binding site. This motion results in the closure of the aqueous cavity on the extracellular side and the opening of a new cavity that exposes the substrate-binding site to the cytosolic side. The substrate can then diffuse out of the binding site into the cytosol.

In the gated-pore mechanism (FIG. 3b), the substrate-binding site of the transporter is also located close to the centre of the membrane but, in this case, the protein uses two distinct gates for transport: one that controls access to the extracellular environment and another that controls access to the cytosol. The two gates cannot open simultaneously, but rather open and close one at a time to enable a substrate to move across the membrane. An intermediate protein conformation occurs when both gates are closed, which leads to an occluded substrate-binding site. In both the rocker-switch and gated-pore mechanisms, the protein is dynamic and undergoes substantial conformational changes, but the substrate stays more or less in the same place (relative to the membrane) until it diffuses out of the binding site at the opposite side of the membrane. Such mechanisms have been described as 'moving-barrier mechanisms'[44].

In the elevator mechanism (FIG. 3c), the substrate does not remain in the same place during the transport cycle. The substrate binds to a domain of the protein that moves

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through the membrane during transport. Similarly to an elevator, the protein domain undergoes a sliding or translational movement through the membrane, but the overall conformation of the domain does not change during movement (it resembles a rigid body). Similarly to the gated-pore mechanism, the elevator mechanism involves two gates that alternate access of the substrate-binding pocket to either side of the membrane, and an occluded intermediate state also occurs during the movement. A scaffold domain is present (FIG. 3c) along which the transport domain can move. The elevator mechanism is considered to be a manifestation of the 'moving-carrier mechanism'[42–46].

The two crystal structures of ECF transporters suggest that they use a different transport mechanism, although it should be noted that the proposed mechanism requires experimental validation. The structures of the isolated S components BioY, RibU and ThiT indicate that substrate binding takes place at a site that is close to the extracellular side of the membrane[23–25] (FIG. 2b), and the loop that connects membrane-spanning segments 1 and 2 (known as Loop 1–2) functions as a gate that controls access[39]. When this gate is closed, the substrate-binding site is occluded. On the basis of the new crystal structures[21,22], it is proposed that the S component that is loaded with the occluded substrate topples over, thereby moving the substrate-binding site to the cytosolic side of the membrane, which is the orientation that was observed for the S components FolT and HmpT in the complete ECF complexes[21,22] (FIG. 3d). Similarly to the elevator mechanism, the S component is proposed to behave as a rigid body during the toppling. In contrast to the elevator mechanism, the S component rotates around an axis in the plane of the membrane instead of making a mostly translational movement. Thus, the transport mechanism that is used by the ECF transporters might be a new manifestation of the 'moving-carrier mechanism', in which the toppling is facilitated by the ECF module. The energetic barrier to rotating a whole protein in the membrane has previously been considered to be prohibitive[43], but such re-orientation is not completely without precedent as it occasionally occurs during membrane protein biogenesis and folding[37,47]. Rotation as part of a catalytic cycle has not been documented before; however, the new structures suggest that ECF transporters might function in this way, although experimental validation of this hypothesis is required.

Transport in the absence of an ECF module. During the proposed translocation cycle, in which the S component topples over, the transported substrate seems to only make contact with the S component. This enables us to speculate on the mechanism by which solitary S components (such as BioY from *Chlamydia* spp.) could mediate transport independently of an ECF module[31,32]. If these BioY proteins could spontaneously topple over in the membrane, they might alternately expose their binding sites to both sides of the membrane. This conformational change might be inefficient in the absence of an ECF module, but it could be sufficient to import the small amounts of biotin that are required for survival. For example, *E. coli* only needs 100–200 molecules of biotin per cell[48], whereas other ECF transporter substrates are usually required in much larger quantities[49]. Alternatively, it is also possible that the solitary BioY proteins are non-specifically assisted by a protein other than an ECF module to enable toppling, or that they form oligomers to facilitate toppling. Oligomerization of *R. capsulatus* BioY has been observed when it is heterologously expressed in *E. coli* cells, although this protein and other solitary S components are monomeric when purified[24,26,35,50].

Coupling of transport to ATP hydrolysis. The ATP-dependence of ECF transporters was observed in the 1970s, long before ABC transporters were discovered[2], and was recently confirmed using purified transporters that had been reconstituted in lipid bilayers[21,29]. The structures of the ECF-transporter complexes, together with the known role for ATP hydrolysis in the function of other ABC transporters, now enables us to speculate on the mechanism of ATP-dependent transport.

In all ABC transporters, ATP binding and hydrolysis takes place at the interface between the ATPase subunits (EcfA and EcfA′ in ECF transporters), where two ATP binding sites are located[28,51] (BOX 1;FIG. 3d). Binding of ATP brings the two subunits into close proximity, whereas they are separated from each other in the absence of ATP[28,51]. The ECF–FolT and ECF–HmpT complexes were crystallized in a nucleotide-free state, with the ATP-binding sites of the EcfA and EcfA′ subunits separated from each other[21,22]. Because all the structural elements that are required for the binding and hydrolysis of ATP are conserved in EcfA and EcfA′, it is probable that ATP binding pulls the two ATPases towards each other, leading to ATP hydrolysis. Similarly to other ABC transporters, it is probable that the associated conformational changes in the EcfA and EcfA′ subunits are propagated to the membrane domain[28]. However, the nature of the conformational changes in the membrane-embedded subunits is currently unclear. It is possible that reorientation of the toppled, substrate-free S component (which was observed in the crystal structures of ECF–FolT and ECF–HmpT) to the upright position is coupled to the binding of ATP to the ECF module. The upright S component would then have access to the extracellular environment to enable the binding of substrate. The subsequent ATP hydrolysis and release of ADP and inorganic phosphate might then be coupled to the toppling of the substrate-loaded S component, thereby enabling the substrate to move from the extracellular side of the membrane to the cytosolic side (FIG. 3d). In addition, some of the free energy that is released by the hydrolysis of ATP could be used to lower the affinity of the S component for the substrate when the toppled state is reached, thereby enabling the release of the substrate. In complete ECF complexes, substrate affinity seems to be much lower than in solitary S components[9,11,24,26], probably as a result of rearrangements in the substrate-binding site upon toppling[21]. A reduction in substrate affinity is likely to be important as it would enable the substrate to diffuse into the cytoplasm.

The role of EcfT. The S components do not directly contact the EcfA and EcfA′ subunits but, instead, the interactions are mediated by EcfT[21,22]. EcfT contains five membrane-spanning α-helices and a conserved cytoplasmic domain with two long α-helices (which are labelled X1 and X2 in FIGS 2a,3d) that form an X-shape and do not span the membrane. Before the crystal structures were solved, the cytoplasmic helices had already been proposed to transmit conformational changes between the ATPases and the membrane subunits[34,52]. Other ABC transporters (such as exporters and type I and type II importers; BOX 1) also make use of helical structures to transmit conformational changes between the ATPases and the membrane domains[53]; however, unlike ECF transporters, in which the helices are found in a single subunit (EcfT), in these ABC transporters both membrane subunits contain a single so-called 'coupling helix'[53], and each membrane subunit contacts a different ATPase subunit via its coupling helix.

The carboxy-terminal ends of the two X-shaped helices of EcfT are anchored to

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the ATPases by two conserved Ala-Arg-Gly motifs[52]; the motif from one helix interacts with EcfA and the other interacts with EcfA′ (REFS 21,22,33). Binding of ATP to EcfA and EcfA′ is predicted to bring the two ATPase subunits into close proximity, which might also push the C-terminal ends of the X-shaped helices of EcfT towards each other as they are anchored via the Ala-Arg-Gly motifs[52] (FIG. 3d). This movement might alter the interaction interface with the S component, which could potentially force the toppled S component (which is oriented parallel to the membrane plane) to return to an upright orientation (that is, perpendicular to the plane of the membrane), which would then result in the exposure of the substrate-binding site to the extracellular side[21,22]. Another intriguing possibility is that conformational changes in the X-shaped helices might lead to dissociation of the S component from the ECF module. The solitary S component might then spontaneously re-orient in the membrane in the upright position. Dissociation of S components during the translocation cycle could explain a puzzling observation that was made in the 1970s[5]: experiments in *Lactobacillus casei* showed that different S components compete for the same ECF module and that this competition depends on the presence of the transported substrate, which suggests that substrate-bound and substrate-free S components have different affinities for the ECF module. Substrate-dependent competition can only be explained if dissociation and re-association takes place, possibly as part of the catalytic cycle.

In addition to the cytoplasmic X-shaped helices, the membrane-embedded domain of EcfT makes extensive contacts with the S component[21,22] via a surface that is almost exclusively hydrophobic. It is possible that the hydrophobic surface of EcfT provides a 'sliding scaffold' for the S component (which is also hydrophobic) to facilitate toppling (FIG. 3d). In group II ECF transporters, such a sliding scaffold would be used by multiple S components, which suggests that there must be conserved elements in the interaction interface between EcfT and the different S components[5,6,29]. Indeed, a conserved sequence motif (Ala-X-X-X-Ala, where X is any amino acid) in the first α-helix of the S components interacts with the X-shaped helical structure of EcfT[21,22] (helices X1 and X2 in FIGS 2a,3d). The Ala-X-X-X-Ala motif had already been discovered when the structures of solitary BioY, ThiT and RibU[24,25] were compared, but is unlikely to be sufficient to provide specificity for an ECF module. Surprisingly, apart from the hydrophobic nature of the interactions, there are no other conserved structural features in the FolT–EcfT and HmpT–EcfT interfaces[21,22]. Therefore, it is still unclear how different S components specifically interact with the same ECF module. Given the divergence in amino acid sequence among the S components (for example, HmpT and FolT share only 17% sequence identity), it is likely that their ability to compete for the same ECF module might also vary.

Outlook

The two crystal structures of complete ECF transporter complexes have generated several hypotheses about the mechanism of transport that must now be tested, such as the toppling mechanism that is proposed here. Spectroscopic techniques, such as electron paramagnetic resonance (EPR)[39,54] and Förster resonance energy transfer (FRET)[55], can be used to assay the dynamics of proteins during catalysis and seem to be suitable for studying the proposed toppling of the S component during the translocation cycle. In addition, crystal structures of ECF complexes in the presence of nucleotides and transported substrates, are likely to provide further mechanistic insight, and may reveal, for example, whether the proposed toppling event is specifically coupled to ATP binding or hydrolysis. Although the molecular basis by which the same ECF module specifically recognizes different S components remains unclear, the crystal structures should facilitate a systematic analysis of the possible interfaces that could be used, which could be tested by mutagenesis of the residues at the interface. Such work should provide insight into a fundamental problem that is poorly understood: how do hydrophobic membrane proteins in the hydrophobic environment of the lipid bilayer specifically recognize each other[56]? Crystal structures of group I ECF transporters should also be pursued in order to investigate possible differences in transport mechanisms between group I and II ECF transporters.

Finally, many pathogenic bacteria, such as *S. aureus, S. pneumoniae* and *M. genitalium* have been shown, or are predicted, to depend on ECF transporters for survival. Therefore, ECF transporters might be suitable targets for novel antibiotics[6]. As the design of small molecules that specifically inhibit protein function depends on the availability of high-resolution structures, structural studies of ECF transporters from pathogens should now be carried out. The high-resolution structures of ECF–FolT and ECF–HmpT from *L. brevis* show that such a task is now feasible.

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Competing interests statement
The author declares no competing interests.

DATABASES
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